

Spearmint (*Mentha spicata* L.) essential oil from tipaza (Algeria): *in vivo* anti-inflammatory and analgesic activities in experimental animal models

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Abstract

Introduction: Although analgesic and nonsteroidal anti-inflammatory drugs (NSAIDs) are usually used to treat a diversity of illnesses, their administration is linked with acute kidney injury and gastrointestinal side effects. The research of new biomolecules and natural products is still needed such as medicinal plants.

Aims: The present research was aimed to investigate, for the first time, the anti-inflammatory and anti-nociceptive effects of spearmint essential oil (SEO) in mouse models of acute inflammation and pain.

Materials and Method: Chemical analysis of SEO was done by gas chromatography. The anti-inflammatory activity was tested using two models of acute inflammation namely carrageenan-induced paw edema and xylene-induced ear edema. Histological examination of both non-inflamed and inflamed tissues was evaluated. The anti-nociceptive activity was tested using the pain model induced by acetic acid.

Results: The main constituent of the SEO was found to be carvone (52.60%). The SEO exhibited a promising anti-inflammatory effect as demonstrated by statistically significant ($p < 0.05$) inhibition of paw volume by 77.24% at the dose of 20 $\mu\text{L}/\text{kg}$ and 65.87% at the dose of 200 $\mu\text{L}/\text{kg}$. Furthermore, topical administration of the SEO inhibited xylene-induced ear edema in comparison with the control group ($p < 0.05$). The higher dose (200 $\mu\text{L}/\text{kg}$) significantly ($p < 0.001$) reduced xylene-induced ear edema which was similar to that observed with positive control (ketoprofen). The pathological analysis of the paws and ears revealed that SEO was capable of reducing cellular infiltration and subcutaneous edema. Else, the SEO produced significant anti-nociceptive activity ($p < 0.001$) at higher dose by inhibiting spontaneous nociception.

Conclusion: These results support the use of SEO in the development of pharmaceuticals for the management of inflammation and pain.

Keywords: Spearmint essential oil; Anti-inflammatory effect; Carvone; Histological analysis; anti-nociceptive activity.

Abbreviations: ANOVA = Analysis of variance; COX-2 = Cyclooxygenase-2; EO = Essential oil; GC-MS = Gas Chromatography-Mass Spectrometry; H&E = Hematoxylin-Eosin; IL = Interleukin; iNOS = Nitric Oxide Synthase; INF- γ = Interferon; IC50 = Median inhibitory concentration; LPS = Lipo-polysaccharide; LTB4 = Leukotriene B4; NIH = National Institute of Health; NIST = National Standard Institute Technology; NSAIDs = Nonsteroidal Anti-Inflammatory Drugs; NV = Neovascularization; *p.o.* = *per os*; PBS = Phosphate-buffered saline; PGE2 = Prostaglandin E2; SEO = Spearmint essential oil; PMN = Polymorphonuclear cells; RT = Retention Times; SD = Standard Deviation; TNF = Tumor Necrosis Factor.

1. Introduction

Inflammation is an imperative biological reaction which happens in answer to a varied range of injurious agents such as physical trauma, bacterial infection, chemicals or any additional physical phenomenon. Inflammation is the complex state of hyperemia from blood vessels with resulting warmth, redness, swelling and discomfort [1]. Immune reaction is important for the body to eliminate and remove hazardous pathogens by way of an acute inflammation [2]. Inflammation usually implicates edema and pain at the location of injury or wound due to discharge of many pro-inflammatory chemical mediators along with the escape of liquid from the vascular tissues due to increase in the penetrability of the vessel barriers, tissue damage, infiltration and migration of inflammatory cells and healing [3]. All kinds of pain, whether it is chronic or acute, initiate from inflammation. During inflammation, several pro-inflammatory chemical factors such as interferon (INF- γ), tumor necrosis factor (TNF), cyclooxygenase-2 (COX-2), interleukins (IL-6 and IL-12), and inducible nitric oxide synthase (iNOS) are synthesized and secreted. The receptors involved in inflammation are similarly stimulated in pain [4].

Non-steroidal anti-inflammatory drugs (NSAIDs), such as diclofenac sodium, are among the most frequently recommended medications due to their regular efficiency in the management and treatment of inflammation, pain and rheumatic complaints [5]. Nevertheless, their administration is linked with undesirable effects at the level of digestive area, ranging from gastrointestinal erosions and severe peptic ulcers to more grave problems, such as over bleeding or full damage of epithelial membrane. Other NSAIDs adverse reactions comprise hepatic, renal and cardiovascular undesirable effects which are correlated to the agent as well as the frequency and period of treatment [6]. Then, to overcome the toxicity of NSAIDs, the investigation of new and promising molecules is still required and the natural products such as aromatic and medicinal plants could lead in the development of new anti-inflammatory and analgesic drugs with fewer side effects.

Numerous species of *Mentha* genus are used in phytotherapy as flavor agents and as herbal medicines for several human therapeutic administrations [7]. Spearmint (*Mentha spicata* L.) is generally cultivated in different regions worldwide to com-

mercially produce its aromatic or essential oil (EO) [8]. Spearmint has several therapeutic properties and health benefits and is used in phyto-medicine as antifungal, diuretic, carminative and antioxidant agents, and for management of colds and flu, hemorrhoids, respiratory tract problems and stomach ache. Interestingly, spearmint essential oils (SEOs) were recognized as rich in oxygenated monoterpenes and were found to have antimicrobial activity [9,10]. It is also a nontoxic and real therapeutic choice for the cure of chemotherapy-induced emesis and nausea in patients [11].

Although several studies have been reported on the chemical composition, antimicrobial and antioxidant activities of spearmint essential oil *in vitro* [7-10], however only few investigations report its pharmacological effects *in vivo*. Based on the above reflections, the present research reports, for the first time, the chemical composition of essential oil obtained from the leaves and stems of spearmint grown in Algeria as well as the *in vivo* anti-inflammatory and anti-nociceptive effects.

2. Material and methods

2.1. Materials

2.1.1. Extraction of Spearmint essential oil

Spearmint (*Mentha spicata* L.) leaves and stems were collected on June 2016 in the Cherrhell region (Tipaza, Algeria). This area is located in the eastern region of Algiers (coordinates 35°52'34"N latitude and 0°17'20"W longitude). SEO was extracted from the aerial part of spearmint with and alembic steam distillation (Extral-Bio company, Chiffa, Blida, Algeria). The whole quantity of fresh plant used was 450 kg, and it was loaded in the still and stacked in layers to allow appropriate delivery of the steam. The process consists of passing water vapor at a high-pressure through an alembic (tank) filled with spearmint. The SEO was stored in a sealed vial, at 4 °C, before use.

2.1.2. Chemicals and drugs

The following drugs and chemicals were used: dimethyl sulfoxide (DMSO), sodium diclofenac, carageenan, xylene, tween 80, isosaline (0.85%) and phosphate-buffered saline (PBS) solutions were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ketum gel® 2.5% (Ketoprofene 60 g,

Laboratoire Menarini, Barcelona, Spain), Spasfon[®] 80 mg (Phloroglucinol, Teva Sante, Paris, France), Ketamile[®] (Ketamine chlorydrate, El Kendi Pharmaceuticals, Algiers, Algeria) and Votrex[®] 50 mg (sodium diclofenac, Hikma Pharmaceuticals, Jordan) were also used.

2.1.3. Animals

Swiss NMRI male mice (25.28±1.75 g) were got from the animal breeding of SAIDAL pharmaceutical company (Algiers, Algeria). The mice were used in agreement with the guiding principles in the care and use of animals agreed by the Council of the American Physiologic Society. The mice were permitted free access to water and food under conditions of optimum light (12 hours light-dark cycle) with the room temperature maintained at 25±3 °C and relative humidity of 45-60%. In anti-inflammatory and analgesic assays, mice were arbitrarily separated into five groups: control, NSAID, and SEO (200, 20, and 2 µL/kg) groups. All experiments were done between 9:00 a.m. and 3:00 p.m. All labors were done in order to diminish animal suffering.

2.2. Methods

2.2.1. Determination of the chemical composition of spearmint essential oil

The chromatographic analysis of the SEO was done using a Shimadzu gas chromatograph (Shimadzu Corporation, Kyoto, Japan), coupled with a Shimadzu mass spectrometer detector QP-5050A. The GC-MS system was equipped with a Tracsil Meta.X5 column, 95% polydimethylsiloxane and 5% polydiphenylsiloxane (Teknokroma S. Coop. C. Ltd., Barcelona, Spain; 60 m × 0.25 mm × 0.25 µm film thickness). Analysis was done using He as carrier gas at a column flow of 0.3 mL/min with a split ratio of 1:200. The oven temperature program was as follow: (a) 80 °C, 0 min; (b) rate of 4 °C/min from 80 to 210 °C and hold for 1 min; (c) rate of 25 °C/min from 210 to 300 °C and hold for 8 min. The temperatures of the injector and detector were 230 and 300 °C, respectively. All chemical compounds were detected and identified using two different techniques: (1) mass spectra (NIST05 spectral library collection), and (2) comparison of their experimental retention indices (RI) with those of the literature. Only fully identified compounds are reported in this study.

2.2.2. In vivo anti-inflammatory activity

2.2.2.1. Carrageenan-induced paw edema in mice

Carrageenan-induced paw edema in mice is a well-established technique of acute inflammation for evaluation of anti-inflammatory molecules. This method was similar to that reported by Mogosan et al. [12]. Mice (six weeks old) weighing about 24–27 g were randomly divided into five groups of six animals; including a control group (vehicle, isosaline NaCl 0.9%); SEO groups (2, 20 and 200 µL/kg), and NSAIDs group (diclofenac sodium, 50 mg/kg), as the standard drug. The designated groups were orally treated with vehicle, SEO, or NSAIDs 40 minutes before λ-carrageenan injection. For induction of inflammation, the mice received an injection of 100 µL of 1% (w/v) suspension of λ-carrageenan in saline, into the mouse's plantar side of the left hind paw after 40 min of the oral administration of the sample. The animals were sacrificed 4 hours later. The difference in weight between right untreated and left treated hind paws was calculated and results are expressed as the increase in paw weight (mg). The percentage inhibition of the inflammatory response was calculated by comparison to the negative control and SEO or NSAIDS groups by using this formula:

$$\% \text{ Inhibition of edema} = \left(\frac{(C_t - C_0)_{\text{Control}} - (C_t - C_0)_{\text{Treated}}}{(C_t - C_0)_{\text{Control}}} \right) \times 100$$

Where $(C_t - C_0)_{\text{control}}$ is the difference in the weight of paw at 4 hours in control animal, and $(C_t - C_0)_{\text{treated}}$ is the difference in the weight of paw at 4 hours in animal treated either with the standard drug or SEO.

At the end of the assay, the mice were euthanized by diethyl ether and the inflamed paws were removed and fixed in 10% formaldehyde solution for histological analysis and examination.

2.2.2.2. Histological examination of mouse paw tissue

To further confirm the inflammatory changes in paw of animals after injection of carrageenan, the mice were sacrificed 4 h after the generation of inflammation. The sub-plantar paw tissue samples were then removed, fixed in 10% neutralized formalin for 24 h, dehydrated with alcohol (ethanol), surrounded in paraffin wax (56 °C) and cut into 5 µm thick sections. Successive sections were stained with haematoxylin and eosin (H&E) stain

to establish the degree of edema and inflammation [13]. The stained sections were examined under a light microscope and the histological modifications were registered with the assistance of a pathologist. The severity of paw tissue inflammation was evaluated by two independent observers blinded to the method protocol.

2.2.2.3. Xylene-induced mice ear edema

Topical inflammation was done using ear edema model according to the method of Boukhatem et al. [13]. Adult Swiss albino mice were arbitrarily divided into five groups, each containing six mice. The acute inflammation was made on the posterior and anterior surfaces of the right ear by the topical application of 20 μ L/ear of xylene. The left ear (untreated) was considered as a control. To determine the topical anti-inflammatory effect, SEO diluted in almond oil was applied topically at different doses (200, 20 and 2 μ L/kg) 40 minutes before the xylene application. Another group was treated only with vehicle (almond oil) and was considered as a control. Ketoprofen (NSAIDs) was used as a reference drug. Four hours after the application of xylene, the animals were sacrificed and two ear punches (6 mm diameter) were taken from both untreated (left) and treated (right) ears and weighed. The weight difference between the right and left ear disks of the same mice was assessed as the intensity of edema. The anti-inflammatory effect was estimated and expressed as a percentage reduction of edema in treated mice compared with the control and calculated using the following formula:

$$\% \text{ Inhibition of topical edema} = \left(1 - \frac{\Delta W_t}{\Delta W_c} \right) \times 100$$

where ΔW_t is the change in weight of ear tissue in the treated mice, and ΔW_c the change in weight of ear tissue in the control mice (vehicle).

2.2.2.4. Histological examination of mouse ear tissue

The subsequent inflammatory response was assessed and monitored by estimation of edema formation and by microscopic observation. For the histological study of cutaneous inflammation, two samples of the swollen ears from the control and the SEO treated groups were removed and fixed in 10% formaldehyde for one week. Then, the fixed ear tissues were implanted in paraffin, routinely processed and sectioned at 5 μ m using a microtome (LEICA RM, Nussloch, Germany). The slices were

mounted on the glass slides, stained with H&E and lastly observed and examined by a pathologist in a blinded way. The ear tissues were observed with a light microscope (Olympus CX41) and graded as minor (+), modest (++), or severe (+++) for inflammation phase. Infiltration and polymorphonuclear (PMN) cells' accumulations and infiltration were also reported [13]. The histological examination was carried out in the laboratory of histopathology, Hospital of Kolea (Tipaza city, Algeria).

2.2.3. In vivo anti-nociceptive activity of SEO using acetic acid-induced writhing test

The anti-nociceptive activity of SEO was investigated using the writhing assay (abdominal constriction test), according to the method of Mogosan et al. [12]. A total number of 30 male mice were divided into five groups. Briefly, mice were treated orally with phloroglucinol (Spasfon 80[®] mg/kg, i.p.) which is considered as a standard analgesic drug. Also, SEO and vehicle (isosaline, NaCl 0.9%) were administered orally 30 min. After that, intra-peritoneal injection of acetic acid (0.6%) was done for the induction of abdominal contractions. The number of abdominal contractions or writhes were counted for each group of mice starting from one minute after the injection of acetic acid up to 10 minutes and expressed as percent protection. The anti-nociceptive activity was estimated as the percentage of inhibition of abdominal contractions between the control and the treated groups (SEO or standard drug) using the following formula:

$$\text{Protection (\%)} = \left(\frac{N_c - N_t}{N_c} \right) \times 100$$

Where N_c is number of writhing in control, and N_t is the number of writhing in test mice.

2.2.4. Statistical analysis

Data obtained in our research was presented as mean \pm SD where each value represents a minimum of 6 mice. One way analysis of variance (ANOVA) was done to evaluate the variability among groups. Significant differences among groups were calculated using Tukey's multiple comparison tests in which the results were compared with that of control group. The results were considered statistically significant at $p < 0.05$. XLSTAT software 2014 for Windows (Addinsoft, Paris, France) was used for all statistical analysis.

3. Results and discussion

3.1. Chemical composition of spearmint essential oil using GC-MS analysis

The EO was extracted by steam distillation from the dried aerial parts of spearmint from the Chercell region (Tipaza, Algeria), and was analyzed by GC-MS (Figure 1 shows a representative chromatogram). The SEO showed a diverse composition with 19 constituents reported in Table I. The most abundant chemical components were oxygenated monoterpenes 64.7%, followed by monoterpene hydrocarbons (28.84%), and sesquiterpene hydrocarbons (6.46%). The main constituents were carvone (52.60%) and limonene (24.99%), followed by 1.8-cineole (7.22%) and *trans*-caryophyllene (3.38%). Other chemical compounds were detected but were less than 3%.

The EO of this Algerian variety of mint can be ascribed to the carvone/limonene chemotype. Actually, there is great variation in the chemical composition of spearmint, cultivated as well as endemic or wild, around the world. Remarkably, our SEO seems to be richer with some main compounds such as eucalyptol (1.8-cineole) and oxygenated monoterpenes. A review of the literature accessible on this theme shows that several articles have previously been published on SEOs chemical composition [10,11]. In addition, our findings about most major chemical constituents of SEO collected from the center part of Algeria are in agreement with previous reports [8,9]. Govindarajan et al. [14] studied the chemical composition of SEO obtained from

Table I Chemical composition of Algerian *Mentha spicata* (L.) essential oil using GC-MS.

RT (min)	Name	%
8.855	α -Pinene	0.66
9.742	β -Pinene	1.97
10.132	β -Myrcene	0.77
10.853	Limonene	24.99
10.899	1.8-Cineole	7.22
11.178	β -Ocimene	0.10
11.324	γ -Terpinene	0.35
11.487	<i>cis</i> -Terpineol	0.16
13.253	Terpineol-4	1.22
13.562	<i>cis</i> -Dihydrocarvone	1.06
13.655	Dihydrocarveol	0.88
14.187	Pulegone	0.38
14.442	Carvone	52.60
15.392	Dihydrocarvyl acetate	0.82
15.858	<i>cis</i> -Carvyl acetate	0.36
16.154	β -Bourbonene	1.87
16.234	β -Elemene	0.96
16.610	<i>trans</i> -Caryophyllene	3.38
17.022	α -Humulene	0.25
	Oxygenated Monoterpenes	64.7
	Monoterpene Hydrocarbons	28.84
	Sesquiterpene Hydrocarbons	6.46

Tamilnadu (India) and reported carvone (48.60%) and limonene (11.30%) as the dominant compounds. Padalia et al. [15] reported the chemical composition of 16 cultivars of Chinese *Mentha* species and revealed that carvone (51.3%–65.1%) and limonene (15.1%–25.2%) are the principal compounds in five cultivars of spearmint. In contradiction with our data, Telci et al. [16] studied chemical composition and antimicrobial effect of SEO and demonstrated that piperitone and pulegone were the main components. Else, El-Sayed et al. [17] confirmed that menthone (32.43%) and eucalyptol (18.79%) were the dominant constituents of the Egyptian SEO. Further, a linalool-rich chemotype (82.8%) was described from Turkey [18].

Usually, the observed changes in chemical composition of SEO, when compared with earlier reports, could be linked to different abiotic or biotic factors comprising several extraction methods, climate and seasonal variations, geographical conditions, relative humidity, agronomic conditions (harvesting time, crop density, organic fertilizers), genotype, stage of the plant growth and processing of plant materials before distillation of the SEO [19].

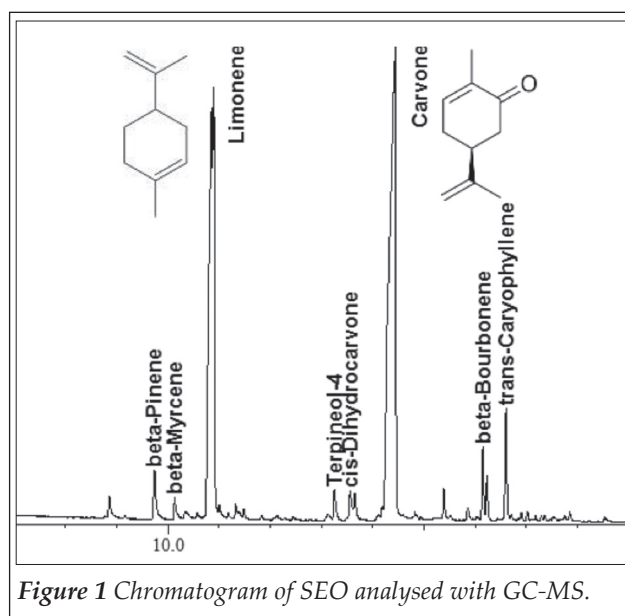


Figure 1 Chromatogram of SEO analysed with GC-MS.

3.2. *In vivo* anti-inflammatory activity

3.2.1. *In vivo* anti-inflammatory activity using carrageenan-induced paw edema test

The anti-inflammatory effect of medicinal herbs has been confirmed and few others refuted. Therefore the present research is carried out with a purpose to screen the effectiveness of anti-inflammatory potential of SEO in experimental animals. The acute inflammation was experimentally made by carrageenan to evaluate the anti-inflammatory property of SEO in mice. Subcutaneous injection of carrageenan produced an increase in animal paw weight due to edema, thus demonstrating acute inflammation.

As shown in [Table II](#), SEO showed an interesting anti-inflammatory potential. At 4 hours after oral administration of SEO, the weight of treated left hind paw was similar for 200 $\mu\text{L}/\text{kg}$ and 20 $\mu\text{L}/\text{kg}$ (3.7 ± 6.3 mg and 0.25 ± 3.48 mg, respectively) with edema inhibition values of 65.87% and 77.24%. This level of edema inhibition was similar to the level detected using 50 mg/kg of the standard reference drug (85.32%). Our findings revealed that treatment with SEO was effective in decreasing the edema induced by carrageenan. However, the drug used as a reference (sodium diclofenac) was more effective in preventing these effects.

The carrageenan-induced paw edema assay is the most commonly used test to determine new anti-inflammatory molecules or drugs. This model is greatly reproducible and has been well recognized as an effective technique to investigate pro-inflammatory cytokine generation and mediators (serotonin, histamine, prostaglandins and bradykinin) in the paw tissue in inflammatory situations [12,20].

Our investigation presents a primary research and additional studies are needed to explain the

effect and mechanism of action of the SEO components. Several studies reported that the anti-inflammatory activities obtained with SEO might be related to some anti-inflammatory molecules such as oxygenated monoterpenes (carvone and eucalyptol) [20,21]. In fact, D-carvone inhibited the animal hind paw edema induced by various phlogistics (histamine, carrageenin, dextran and bradykinin) in a dose-dependent manner [20].

In addition, it has been reported that mint oil also prevents the inflammatory consequences of Lipo-polysaccharide (LPS), together with inhibition of prostaglandin E2 (PGE2), interleukin-1 (IL-1) and leukotriene B4 (LTB4) production by LPS-stimulated human monocytes [22,23].

3.2.2. Histological examination of the mice paws after injection of carrageenan

To confirm the anti-inflammatory effect of SEO histologically, paw biopsies were harvested 4 h after carrageenan had been injected. Paw tissues from all the groups of animal were examined by H&E staining. The control groups not induced by inflammation showed normal tissue ([Figure 2A](#)) with no inflammation, tissue destruction or swelling phenomenon in the paws of normal mice. On the other hand, the animal injected with carrageenan ([Figure 2C](#)) showed a strong edema and inflamed cavities in the paw tissue, characterized by congestion of blood vessels, dermal and epithelial tissue with an important number of infiltrated inflammatory cells detected in the paw tissue. The cell types were mostly neutrophils, which describe acute inflammation.

As for the positive ([Figure 2B](#)) and SEO groups ([Figure 2D, E and F](#)), edematous condition was clearly decreased by treatment with NSAID (diclofenac sodium, 50 mg/kg) and SEO (2, 20, and

Table II *In vivo* Effects of the spearmint EO (2, 20 and 200 $\mu\text{L}/\text{kg}$) and diclofenac sodium (50 mg/kg) on carrageenan-induced paw edema in mice.

Treatment	Weight (mean, mg) \pm SD			% Inhibition of edema
	Left paw	Right paw	Edema weight #	
Negative control (Vehicle)	140.2 \pm 18.72	129.3 \pm 11.68	10.9 \pm 15.19 ^B	/
Positive control (NSAID)	132.4 \pm 8.99	130.8 \pm 10.00	1.6 \pm 3.21 ^A	85.32
SEO 200	134.1 \pm 13.38	130.4 \pm 17.54	3.7 \pm 6.30 ^{AB}	65.87
SEO 20	139.7 \pm 12.39	137.2 \pm 15.07	2.5 \pm 3.48 ^{AB}	77.24
SEO 2	140.1 \pm 12.08	132.9 \pm 18.34	7.2 \pm 14.03 ^{AB}	33.57

Groups of mice (n = 6/group) were pretreated with vehicle (NaCl, 0.9%), NSAID: non-steroidal anti-inflammatory drugs (Sodium diclofenac, 50 mg/kg, *p.o.*). SEO: Spearmint essential oil at doses of 2, 20, and 200 $\mu\text{L}/\text{kg}$ (*p.o.*) # Means within the same column followed by the same capital letter are not significantly different ($p > 0.05$) according to ANOVA one way analysis followed by Tukey's *post hoc* multiple comparison test.

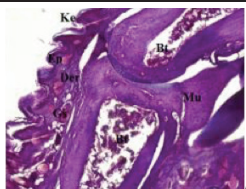
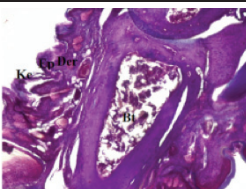
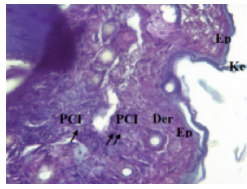
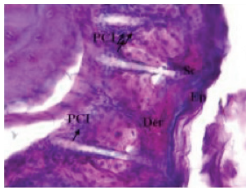
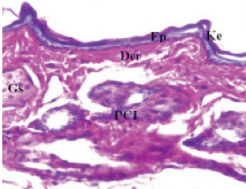
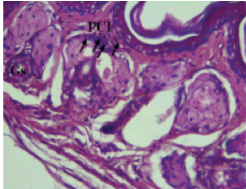
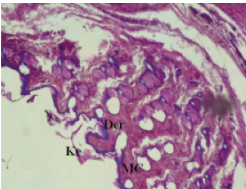
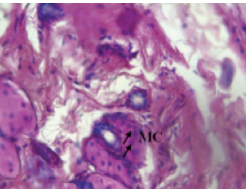
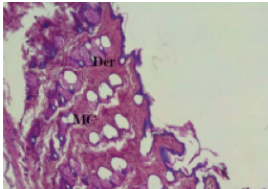
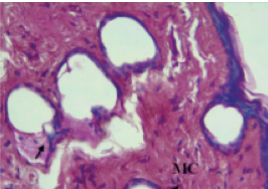
	
Right paw (control) (Gx10) = Edema (-); inflammatory cell infiltration (-), inflammation phase (-).	
	
Positive control treatment (sodium diclofenac) (Gx40) Edema (±); inflammatory cell infiltration (+), inflammation phase (±).	Negative control = λ-carrageenan group (Gx40) Edema (+++); inflammation phase (+++); inflammatory cell infiltration (+++) in epidermal and dermal layers, muscle and cartilage.
	
SEO treatment (2 µL/Kg) (D1 x10; D2 x10) Edema (±); inflammatory cell infiltration (+), inflammation phase (±).	
	
SEO treatment (20 µL/Kg) (E1 x10; E2 x40) Edema (±); inflammatory cell infiltration (+), inflammation phase (±).	
	
SEO treatment (200 µL/Kg) (F1 x10; F2 x40) Edema (±); inflammatory cell infiltration (+), inflammation phase (±).	
Ke: keratin; Ep = epidermal layer; SC = subcutaneous layer; Bt = bone tissue; Mu = muscle fibers; Gs = sebaceous gland; PCI = polymorphonuclear cells infiltration; MC = mononuclear cells; Ed: edema.	

Figure 2 Histopathological examinations on λ-carrageenan-induced paw tissue swelling, edema and neutrophil infiltration. Light photomicrographs of the H&E stained paw tissue in the (A) control group (right paw), (B) NSAIDs group, (C) λ-carrageenan group, (D) SEO (2 µL/kg), (E) SEO (20 µL/kg) and (F) SEO (200 µL/kg) groups. Paws were harvested 4 h after injection of carrageenan and subjected to histochemical staining.

200 µL/kg) as well as a decrease in the infiltration of inflammatory cells. At higher dose of SEO, treated animals showed a better effect in decreasing of neutrophils as compared with standard drug (Figure 2F vs 2C).

Our findings clearly establish the property of SEO within the target tissue, giving additional confirmation that SEO reduces carrageenan-induced paw edema. Mogosan et al. [12] stated that the treatment of SEO inhibited the edema and decreases the cellular infiltrates probably by decreasing the inflammatory chemical mediators.

The histopathological findings also substantiate with the paw edema analyses. Histopathological examinations indicated that edema formation and, inflammatory cell infiltration were obviously suppressed in the animal treated with the SEO. These data support the results obtained from the paw edema test and confirm the anti-inflammatory action of SEO against acute inflammation.

The use of current NSAIDs is frequently associated with severe and different side effects. Hence alternative therapeutic molecules are required. Few studies have been initiated around the world into researching, evaluating and analyzing the local medicinal plants with anti-edematous values. In spite of the medicinal potential of SEO, its effect on inflammation has not been previously reported in detail. Therefore, in the present study, we screened and confirmed for the first time the anti-inflammatory efficacy of SEO against carrageenan induced paw edema followed by histological analysis.

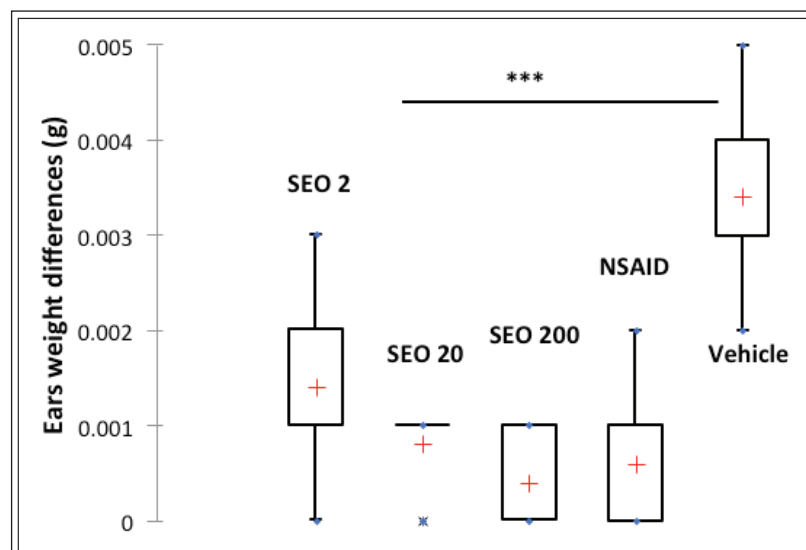
3.2.3. *In vivo* anti-inflammatory activity using xylene-induced ear edema assay

The anti-inflammatory effect of SEO was further assessed by the inhibition of xylene-induced ear edema. As shown in Table III, topical application of xylene in the control group caused

Table III Effects of the SEO (2, 20 and 200 $\mu\text{L}/\text{kg}$) and Ketoprofen (5 mg/ear) on xylene-induced ear edema in mice.

Treatment	Weight (mean, mg) \pm SD			% inhibition of edema
	Right ear	Left ear	Edema weight #	
Negative control (Vehicle)	9 \pm 0.44	12.6 \pm 1.34	3.6 \pm 1.14 ^B	-
Positive control (NSAID)	7.4 \pm 1.51	7.8 \pm 1.09	0.4 \pm 1.14 ^A	88.88
SEO 200 $\mu\text{L}/\text{kg}$	8 \pm 0.83	8.2 \pm 0.83	0.2 \pm 0.54 ^A	94.44
SEO 20 $\mu\text{L}/\text{kg}$	8 \pm 0.54	8.4 \pm 0.54	0.4 \pm 0.44 ^A	88.89
SEO 2 $\mu\text{L}/\text{kg}$	7.4 \pm 0.89	8.8 \pm 0.83	1.4 \pm 1.14 ^A	61.11

Groups of mice ($n = 6/\text{group}$) were pretreated with vehicle, NSAID: non-steroidal anti-inflammatory drug (Ketoprofen 2.5%). SEO: Spearmint essential oil at doses of 2, 20, and 200 $\mu\text{L}/\text{kg}$ (topically). # Means within the same column followed by the same capital letter are not significantly different ($p > 0.05$) according to ANOVA one way analysis followed by Tukey's *post hoc* multiple comparison test.

**Figure 3** Topical anti-inflammatory effect of SEO in xylene-induced ear edema.

Vehicle: control group (mice treated with almond oil topical application); SEO: mice treated with spearmint essential oil topically at different doses (2, 20, and 200 $\mu\text{L}/\text{kg}$) per ear; NSAID: mice treated with non-steroidal anti-inflammatory drugs (ketoprofen 2.5%). Values represent the mean \pm SEM. *** significant difference ($p < 0.001$) according to ANOVA one-way analysis followed by Tukey's *post hoc* multiple comparison tests.

a marked increase in the weight of the ears. However, the topical administration of the SEO (200, 20 and 2 $\mu\text{L}/\text{kg}$), 40 minutes before xylene application, decreased the development of ear edema. The inhibitory effect of the oil was similar to the inhibition caused by ketoprofen (Figure 3). Treat-

ment of the mice with a high dose of SEO suppressed xylene-induced ear edema, reducing swelling by 94.44% ($p < 0.001$). The data indicate that SEO possesses inhibitory effects against acute inflammation.

Our findings revealed that SEO can markedly prevent the formation of xylene-induced ear edema. Mice ear edema may comprise the release of pro-inflammatory chemical mediators, increasing vascular permeability, leukocytes infiltration, plasma leakage and the production of cytokines such as interleukin 1 β , tumor necrosis factor alpha (TNF α) [24]. SEO may produce the anti-inflammatory action by influencing the actions of the above mediators [22,25].

NSAIDs are usually among the most used drugs in the treatment of inflammation. Unfortunately, the use of NSAIDs is restricted by several adverse effects and about 20% of patients will develop gastric ulcer [12]. Our study demonstrated that SEO showed similar effects with NSAIDs on the two models of inflammation (carrageenan and xylene).

Table IV Study of anti-nociceptive activity of SEO by acetic acid-induced writhing test

Treatment	Number of writhing (Mean \pm SD) #	Inhibition (%)
Negative control (acetic acid)	77.8 \pm 5.40 ^B	-
Positive control (Phloroglucinol)	55.6 \pm 9.09 ^A	28.53 ***
SEO 200 $\mu\text{L}/\text{kg}$	59.4 \pm 5.89 ^A	23.65 **
SEO 20 $\mu\text{L}/\text{kg}$	65.2 \pm 3.96 ^A	16.19 *
SEO 2 $\mu\text{L}/\text{kg}$	65.24 \pm 3.96 ^{AB}	16.14 ^{ns}

SEO: mice treated with spearmint essential oil at different doses (2, 20, and 200 $\mu\text{L}/\text{kg}$, $p.o$). Values are expressed as mean \pm SD ($n = 6$, per group); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. # Means within the same column followed by the same capital letter are not significantly different ($p > 0.05$) according to ANOVA one way analysis followed by Tukey's *post hoc* multiple comparison test.

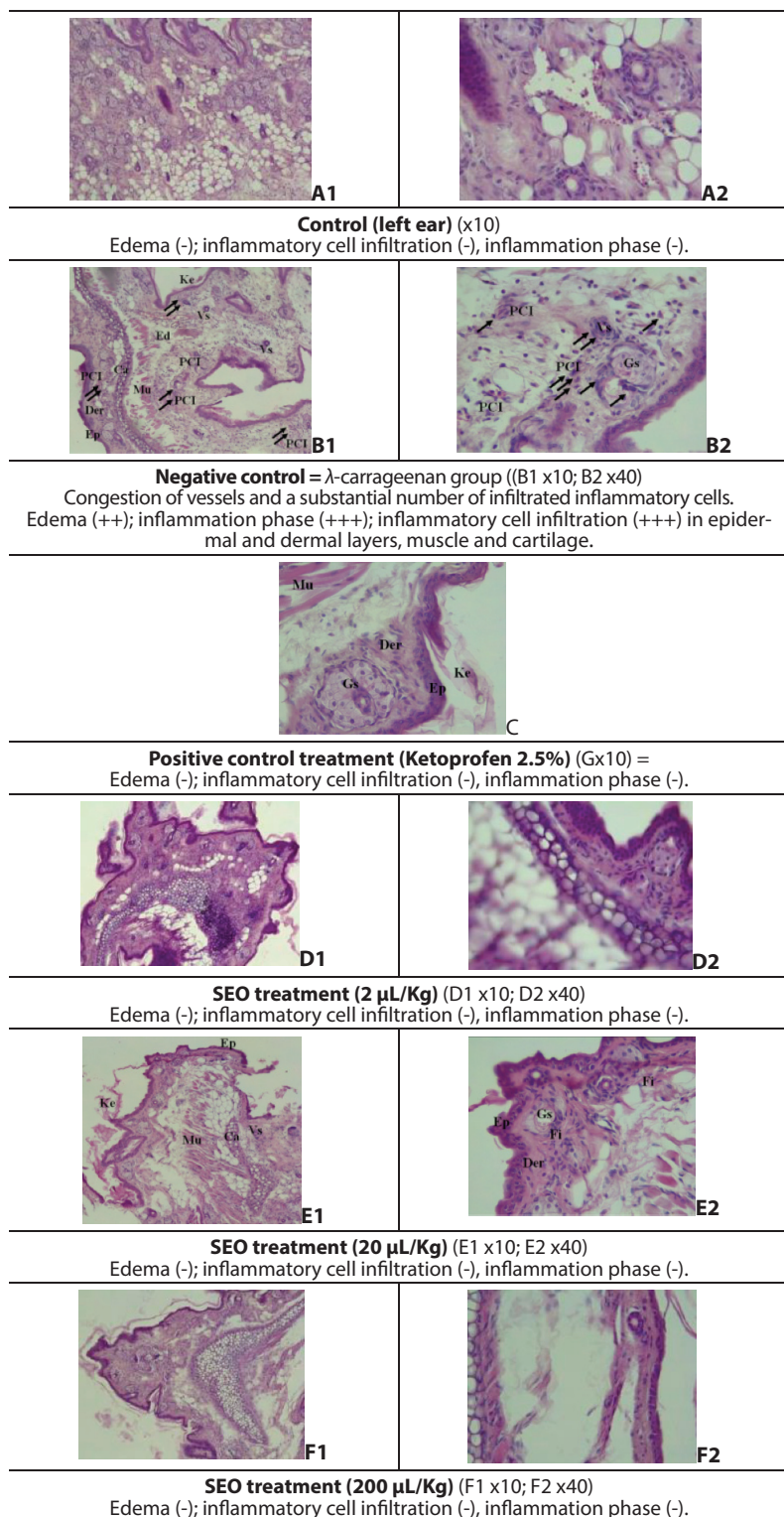


Figure 4 Pathological examination of ear tissues after topical administration of xylene. Histopathology sections of mice ear biopsies showing keratin, epidermal, dermal, muscle, and cartilage layers. Hematoxylin & Eosin stained sections were scored as mild (+), modest (++) and severe (+++) for edema and substantial inflammatory polymorphonuclear cell infiltration (PCT) in the dermis inflammation phase.

(A) Normal Ear; (B) Control: Topical Application of xylene (20 µL/ear) showed induced inflammatory lesion with edema and infiltration of polymorphonuclear leukocytes; (C) Ketoprofen decreased the indicated changes; Topical Application of the SEO at doses of 2 (D), 20 (E) and (F) 200 µL/kg after 4 h of topical xylene was able to decrease epidermis thickness, edema, and infiltration of polymorphonuclear leukocytes. Ke: keratin; Ep: epidermal layer; Bo: bone tissue; PCT: polymorphonuclear cells infiltration; Ed: edema; Mu: muscle.

3.2.4. Examining the mouse ear tissue histology

Anti-inflammatory effect of SEO on the carrageenan test led us to determine this effect in another neurogenic inflammatory assay (xylene-induced ear edema). We investigated H&E stained ear sections from xylene-induced ear edema (Figure 4). By histological comparison, topical application of SEO decreased paw thickness and associated pathological indicators (Figure 4D, E and F) comparable to the positive control (ketoprofen gel) (Figure 4C).

The ear edema model induced by xylene has certain advantages in the determination of NSAIDs and has better predictive values in the screening of anti-inflammatory new molecules [23]. Xylene-induced swelling is followed by innate immunity reaction of the skin, a cytotoxicity response of activated T cells followed by the migration of polymorphonuclear leucocytes which enhance inflammation and swelling of the ear. The pathological examination of inflamed tissues (paws and ears) showed that SEO inhibited infiltration of polymorphonuclear leucocytes into the site of inflammation. Therefore, another option is that SEO exerts its anti-edematous action partly through the inhibition of polymorphonuclear cell infiltration [22,23,25]. In addition, the chemical composition of SEO revealed the presence of high quantity of oxygenated terpene compounds such as carvone, menthol and eucalyptol. Literature review showed that the presence of these compounds in the SEO may be the main reason of its important anti-inflammatory property [21].

3.3. In vivo effect of SEO on acetic acid-induced writhing response

Acetic acid induced writhing experiment was done to evaluate the peripheral analgesic property of SEO. It is well known that acetic acid in some

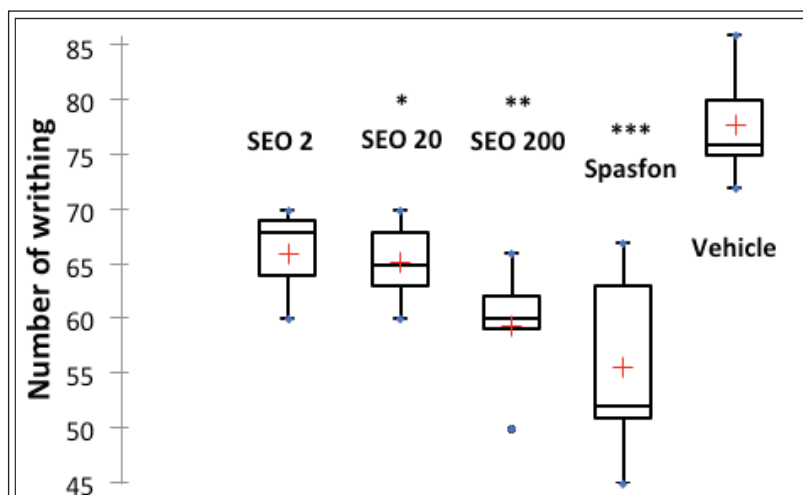


Figure 5 Anti-nociceptive activity of SEO in the acetic acid induced writhing test *in vivo*.

The effect of the SEO (2, 20 and 200 $\mu\text{L}/\text{kg}$, *i.p.*), and Phloroglucinol (Spasfon® 80 mg/kg , *i.p.*) in the acetic-acid-writhing-induced nociception test in mice. Values are expressed as mean \pm SD ($n = 6$, per group); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ significantly different from the control group, according to ANOVA, followed by Tukey's test.

way is responsible for secretion of endogenous mediators thereby stimulating the neurons responsible for pain perception, which are receptive to NSAIDs [26]. In this research, SEO showed a significant *in vivo* analgesic effect using the acetic acid-induced writhing test. Intraperitoneal (*i.p.*) injection of acetic acid produced 77.8 ± 5.40 writhes in the solvent control group (Table IV and Figure 5). The writhing response was significantly decreased by pretreatment with 200 $\mu\text{L}/\text{kg}$ of SEO (77.8 ± 5.40 ; $p < 0.01$). Standard group which received Spasfon (Phloroglucinol) showed 28.53% ($p < 0.001$) of inhibition in writhing movement.

The analgesic properties of mint species have been studied and demonstrated by several authors [12,27]. In this study, we evaluated the anti-nociceptive effect of SEO using the acetic-acid induced pain model. The oral administration of SEO produced significant inhibition of the acetic acid-induced abdominal writhing in dose dependent manner in the mice, but the inhibition was similar to that produced by Phloroglucinol only at a higher dose (200 $\mu\text{L}/\text{kg}$). These data suggest that SEO may produce peripheral analgesic activity by inhibiting the chemical mediators and/or cytokines (histamine, serotonin, bradykinin, cytokines, and eicosanoids). These mediators stimulate an increase of vascular permeability as well as reduce the threshold of nociception and stimulate the nervous terminal of nociceptive fibers [7,23]. The anti-inflammatory and anti-nociceptive activities of SEO may be related to the

synergy interactions of its major or minor chemical components such as carvone, limonene and eucalyptol [28].

4. Conclusion

Our findings clearly confirmed that SEO has a potent peripheral analgesic activity and also demonstrated systemic and local anti-inflammatory properties in carrageenan and xylene models and these preliminary data could provide some credit for the potential use of spearmint as a bioactive agent for the treatment-prevention of inflammatory and painful conditions. Additional cellular and molecular assays will be done to investigate its real mechanisms of SEO including its active components such as car-

vone and limonene, alone or in combination, and to characterize the receptors involved in the anti-nociceptive and anti-inflammatory effects. A systemic study is required to produce nutraceuticals drug from SEO for treating various health problem of human kind.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethics approval

The experimental assay was agreed and approved by the European guidelines for animal handling and experiments.

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Authors' contributions

Study concept and design: SK, MNB and MAF; Experiments: SK, AB and MNB; Analysis and interpretation of data: SK, AB and FB; Drafting of the manuscript: MNB, FB, and WS; and Critical revision of the manuscript for important intellectual content: SK and WS. All authors critically revised the article for important intellectual content and approved the final version.

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